1.0 INTRODUCTION AND RATIONALE FOR THE USE OF *IN VITRO* ER BINDING ASSAYS

1.1 Introduction

1.1.1 Historical Background of *In Vitro* Endocrine Disruptor Assays and Rationale for Their Development

It is well known that small disturbances in endocrine function, especially during highly sensitive stages of the life cycle (e.g., fetal and prepubertal development), can lead to significant and lasting effects on the exposed organism (Kavlock et al., 1996; U.S. EPA, 1997; NAS, 1999). In recent years, evidence has been accumulating to suggest that exposure to natural and anthropogenic substances in the environment may adversely affect the endocrine and reproductive systems of mammals, fish, reptiles, amphibians, and birds. Substances that cause such effects are classified as "endocrine disruptors". Disruption of the endocrine system has been demonstrated in laboratory animals and documented in wildlife (Ankley et al., 1998). For example, male fish caught in rivers in many regions of the United States have high levels of vitellogenin, a female-specific protein (Purdom et al., 1994; Folmar et al., 1996), and female mosquitofish living in streams in which pulp mill effluents containing steroidal substances have been discharged possess male gonadal structures (Bortone et al., 1989). The degree to which humans are affected by endocrine disruptors is unknown, although there are reports that these substances might be contributing to increasing incidences of breast, prostate, and testicular cancers (Glass and Hoover, 1990; Adami et al., 1994; Toppari et al., 1996) and to precocious puberty, hypospadias, and decreased sperm counts (Carlsen et al., 1992; Sharpe and Skakkabaek, 1993). However, other investigators have concluded that there is no evidence for endocrine disrupting effects in humans (Safe, 2000; Barlow et al., 1999).

In 1996, the U.S. Congress responded to societal concerns by passing legislation requiring the U.S. EPA to develop a screening and testing program, using appropriately validated test methods, to detect potential endocrine disruptors in pesticide formulations (the Food Quality Protection Act; FQPA) (P.L. 104-170), and in drinking water (the 1996 amendments to the Safe Drinking Water Act; SDWA) (P.L. 104-182). As a result of these mandates, the U.S. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to provide advice on how to best design a screening and testing program for identifying endocrine

disruptors. In August 1998, EDSTAC issued a report recommending that the U. S. EPA evaluate both human and ecological (wildlife) effects; examine effects to estrogen, androgen, and thyroid hormone-related processes; and test both individual substances and common mixtures (U.S. EPA, 1998a). In December 1998, based on these recommendations, the U.S. EPA proposed the EDSP (U.S. EPA, 1998b). In 1999, the EDSP and its proposed approach to screening for endocrine disruptors were endorsed by the U.S. EPA Science Advisory Board (SAB) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP), which also made a number of recommendations concerning the proposed approach (U.S. EPA, 1999).

The EDSP proposes a two-tiered approach for screening and testing. Tier 1 is comprised of *in vitro* and *in vivo* assays and is designed as a screening battery to detect substances capable of interacting with the estrogen, androgen, or thyroid hormone systems. Tier 2 is comprised of *in vivo* assays only and is designed as a testing battery to (1) determine whether an endocrine-active substance (identified in Tier 1 or through other processes) causes adverse effects in animals; (2) identify the adverse effects; and (3) establish a quantitative relationship between the dose and the adverse effect (U.S. EPA, 2000).

The EDSP's proposed Tier 1 screening battery includes the following assays:
In vitro assays:

- ER binding/transcriptional activation (TA) assay
- AR binding/TA assay
- Steroidogenesis assay with minced testis

In vivo assays:

- Rodent 3-day uterotrophic assay (subcutaneous dosing)
- Rodent 20-day pubertal female assay with enhanced thyroid endpoints
- Rodent 5-7 day Hershberger assay
- Frog metamorphosis assay
- Fish gonadal recrudescence assay

The alternative Tier 1 assays include:

- Placental aromatase assay (*in vitro*)
- Modified rodent 3-day uterotrophic assay with intraperitoneal dosing (*in vivo*)
- Rodent 14-day intact adult male assay with thyroid endpoints (*in vivo*)
- Rodent 20-day thyroid/pubertal male assay (*in vivo*)

According to the EDSP, the Tier 1 assays should:

- Detect all known modes of action for the endocrine endpoints of concern;
- Maximize sensitivity to minimize false negatives, while permitting a to-be-determined level of false positives;
- Include a sufficient range of taxonomic groups among the test organisms to reduce the likelihood that important pathways for metabolic activation or detoxification of the test substances are not overlooked; and
- Incorporate sufficient diversity among the endpoints and assays to permit conclusions based on weight-of-evidence considerations.

The proposed Tier 2 testing battery includes the following *in vivo* assays:

- Two-generation mammalian reproductive toxicity assay
- Avian reproduction assay
- Fish reproduction assay
- Amphibian reproduction and developmental toxicity assay
- Invertebrate reproduction

The alternative Tier 2 assays include:

- Alternative mammalian reproductive test
- One-generation mammalian reproduction toxicity test

According to the EDSP, the Tier 2 assays should:

- Encompass critical life stages and processes in mammals (equivalent to humans), fish, and wildlife;

- Encompass a broad range of doses and the administration of the test substance by a relevant route of exposure; and
- Provide a comprehensive profile of biological consequences of substance exposure and relate such results to the causal dose and exposure.

Two proposed *in vitro* components of the Tier 1 screening battery are ER binding/TA assays, and AR binding/TA assays. The primary rationale for inclusion of *in vitro* assays in the EDSP Tier 1 screen is that they:

- Are suitable for large-scale screening;
- Are based on well-elucidated mechanisms of action; and
- Measure specific endpoints.

The Tier 1 assays are informative with regard to the mechanism of action of the presumptive endocrine disruptor and provide guidance for prioritization for further testing. Due to their sensitivity, these *in vitro* tests should permit the identification of an active substance(s) within a complex mixture. TA assays have an advantage over binding assays because they can measure if there is a biological response to receptor binding (i.e., RNA transcription) and thus, unlike binding assays, can distinguish between an agonist (a substance that mimics the action of endogenous hormones) and an antagonist (a substance that binds to a receptor without initiating a biological response, blocking the action of endogenous hormones) (U.S. EPA, 1998b). However, it needs to be emphasized that these *in vitro* assays cannot be used to predict the risk for an adverse health effect in humans or wildlife. Binding assays only measure the physical binding of a substance to the receptor while TA assays infer, but do not prove, that an adverse health outcome can occur *in vivo*.

As part of the validation process for the proposed EDSP assays, the U.S. EPA is supporting an effort to prepare a series of BRDs on the Tier 1 *in vitro* ER binding, AR binding, ER TA, and AR TA screening assays. Other EDSP-proposed assays will be evaluated through other organizations (e.g., the U.S. EPA and the Organisation for Economic Co-operation and Development [OECD]). The objectives of each BRD are to:

- Provide a comprehensive summary of the available published and publicly available unpublished data on the scientific basis and performance of the identified assays;
- Identify available assays that might be considered for incorporation into the EDSP;
- Assess their effectiveness for identifying endocrine-active substances;
- Develop minimal procedural standards for acceptable ER and AR binding and TA assays; and
- Provide a list of candidate substances for future validation studies.

1.1.2 Prior or Proposed Peer Reviews of *In Vitro* ER Binding Assays

Although there has been extensive research conducted in the past few years to develop new and improved *in vitro* assays to identify substances with ER binding and transcriptional activity, there have been no formal peer reviews of the validation status of such assays. This BRD has been prepared for an upcoming ICCVAM expert evaluation of the validation status *in vitro* ER binding assays, in concert with reviews of ER TA assays and *in vitro* AR binding and TA assays.

1.2 Scientific Basis for the Proposed Tier 1 *In Vitro* ER Binding Assays

1.2.1 Purpose for Using *In Vitro* ER Binding Assays

In vitro ER binding assays are designed to identify substances (ligands) that bind to the ER and that might act as an estrogenic agonist and cause estrogenic effects, or interfere with normal estrogen activity *in vivo* by acting as an antagonist. The assays can be divided into two mechanistic categories: those that measure binding to the receptor and those that measure transcriptional activation subsequent to binding to the receptor. Although receptor binding assays detect both agonists and antagonists, they do not distinguish between the two. In contrast, TA assays can be designed to distinguish between agonists and antagonists.

Binding of the natural ligand, 17β-estradiol, to the ER is a prerequisite for the induction of many subsequent estrogenic effects, such as induction of cell proliferation in the uterus, and maintenance of bone and the cardiovascular system. The binding affinity of a xenobiotic substance for the ER determines how well it will compete with 17β-estradiol. *In vitro* ER competitive binding assays are generally performed by quantifying the ability of substances to compete with 17β-estradiol for binding. However, ER binding alone is not sufficient to indicate

or predict subsequent cellular effects. For this reason, *in vitro* ER binding assays will be used in conjunction with other *in vitro* and *in vivo* assays for Tier 1 screening. Results from such assays will be used in a weight-of-evidence approach to select substances for Tier 2 testing.

1.2.2 Development of *In Vitro* ER Binding Assays: Historical Background

The foundation of current *in vitro* assays for detecting the ability of substances to bind to the ER can be traced back to the mid-1960s when the receptor was first isolated. At that time, procedures were developed not only for isolation of the receptor but also for the measurement of 17β-estradiol binding and the competitive binding of other substances to the receptor.

The ER was first identified, isolated, and characterized as a protein by Toft and Gorski (1965; 1966) and Noteboom and Gorski (1965) from the soluble fraction of the rat uterus using radiolabeled 17β-estradiol. In these studies, it was shown that the synthetic estrogen diethylstilbestrol competed with 17β-estradiol for binding, but that the non-estrogenic hormones, testosterone and corticosterone, did not bind the ER, and 17α-estradiol was partially inhibitory. Noteboom and Gorski (1965) also initiated the use of radiolabeled (with tritium, [^{3}H]) 17β-estradiol for measuring receptor binding affinity and showed the response to be stereospecific. These studies were extended by Toft et al. (1967), who showed that a cell-free system derived from rat uterine tissue had the same estrogen-binding properties as were found at physiological concentrations *in vivo*. A Scatchard plot (Scatchard, 1949) was used to determine the dissociation constant of 17β-estradiol for the ER and the number of binding sites in the tissue preparation. The size of the ER was subsequently estimated as 53 kDa. The estimated dissociation constant for 17β-estradiol was $7x10^{-10}$ M. Notides (1970) demonstrated that the 17β-estradiol dissociation constants for receptors isolated from rat uterus and anterior pituitary were similar ($1.55x10^{-9}$ compared to $1.40x10^{-9}$ M) and that the responses of these receptors to estrogenic antagonists were essentially identical.

The translocation of the ER complex from the cytosol into the nucleus and its interaction with chromatin was suggested by the work of Shyamala and Gorski (1968) and Jensen et al. (1968). Gorski et al. (1968) hypothesized that the translocated ER complex had DNA-regulatory activity. Clark and Gorski (1969) used a cell-free system to demonstrate that the ER complex bound

equally well to the "nuclear pellet" derived from the uterus, which contains ER, to the kidney, which lacks ER, and to glass pellets. This observation demonstrated that there are no specific nuclear (as opposed to DNA) receptors for the complex.

Between 1965 and 1971, a number of *in vitro* methods were developed to measure the binding of 17β-estradiol and other substances to the ER. Hähnel (1971) and Jungblut et al. (1972) evaluated a number of these *in vitro* methods using cytoplasmic ERs isolated from calf uteri and human breast cancer tissue. They concluded that the dextran-coated charcoal, Sephadex chromatography, and agar electrophoresis methods for the separation of the receptor-bound ligand from unbound, radiolabeled 17β-estradiol were suitable for routine use and had equivalent sensitivities. However, Jungblut et al. (1972) concluded that the dextran-charcoal procedure would be the most suitable because its labor, time, and cost requirements were the lowest of the three methods. Hähnel (1971) and Shafie and Brooks (1979) evaluated the effects of other protocol factors on the binding of 17β-estradiol to the ER and the measurement of unbound fraction. The factors evaluated included pH, storage time of the cytosolic preparation, time and temperature of incubation of 17β-estradiol with the ER, 17β-estradiol concentration, sulfhydryl blocking reagents, protein concentration of the cytosol, and the competitive absorption of estrogen to the charcoal. Erdos et al. (1970) developed a hydroxyapatite (HAP)-column binding procedure that was able to distinguish 17β-estradiol binding to high-affinity versus low-affinity receptor sites.

In the late 1960s and early 1970s, it became apparent that a competitive *in vitro* binding assay would be useful. In such an assay, an ER that contains bound 17β-estradiol is challenged with other substances to determine if these substances alter its binding. One of the earliest studies was performed by Korenman (1970), who measured the comparative binding affinities of natural and synthetic steroids to rabbit cytosol and compared the results with data from an *in vivo* rodent uterotrophic assay. The correlation between the *in vitro* and *in vivo* responses was considered acceptable. The authors noted that the *in vitro* ER binding assay offered many advantages, but that it could not distinguish between agonists and antagonists.

ER binding assays are most often conducted with a cell-free ER preparation obtained from estrogen-responsive tissues or cells. The current procedures used to isolate ER are essentially the same as those used in the late 1960s and early 1970s. Traditional techniques to measure competitive binding are routinely used as well, including the use of dextran-coated charcoal and HAP to separate receptor-bound ligand from free ligand. Although ER binding assays have changed very little over their 30 plus years of use, some of the newer procedures have incorporated more recently developed technology, including the use of recombinant ER proteins in place of ER isolated from tissues or cells (Matthews and Zacharewski, 2000) and measurement of fluorescence polarization (FP) equilibrium binding in place of the measurement of radioactivity (Bolger et al., 1998). The ER binding assays, as currently performed, are described in detail in **Section 2.**

The procedures used to calculate the binding parameters are essentially variations on the method published by Scatchard (1949), who developed models for the binding of small molecules to proteins and for extrapolating binding data. Puca and Bresciani (1968) used Scatchard's procedure to estimate the number of ER binding sites and the 17β-estradiol association constant in isolated calf uterus tissue. In a "Scatchard plot", a straight line indicates that a single class of binding site is present; if competing binding sites are present, the line will deviate from linearity. The intercept on the abscissa indicates the number of binding sites available; the association constant is the ratio of the intercepts on the abscissa and ordinate (Puca and Bresciani, 1968). Scatchard plots are widely used in receptor binding studies.

Baulieu and Raynaud (1970) proposed using an alternative procedure for approximating the binding parameters of small molecules in protein mixtures. They developed a nonlinear function by plotting the log of the bound fraction to the log of the total ligand, and demonstrated that this procedure was able to quantitatively distinguish between specific and nonspecific binding in a tissue extract that contained a mixture of specific and nonspecific receptors.

The ER binding assays measure the affinity of radiolabeled 17β-estradiol for the ER (K_d), the affinity of the unlabeled ligand for the ER (K_i), and the concentration at which the unlabeled ligand displaces half the specific binding of radiolabeled 17β-estradiol to the ER (IC_{50}). The K_d,

which is measured in concentration units, is the equilibrium dissociation constant of the 17β-estradiol-ER complex and represents the concentration of 17β-estradiol that will bind to half the binding sites at equilibrium in the absence of competitors. A low K_d represents high affinity and a high K_d represents low affinity. The K_i is the analogous constant for the unlabeled ligand. The IC_{50} values depend on a number of factors, such as the specific assay system used, binding affinity of the unlabeled ligand for the ER, labeled 17β-estradiol concentration, ER concentration, and experimental conditions (e.g., pH, exposure duration). In *in vitro* ER binding assays, there are substances that, because of biological inactivity, low solubility, or other considerations, do not decrease the binding of labeled, bound 17β-estradiol by at least 50%. The IC_{50} values for these substances are often reported as being greater than the highest concentration tested or they are classified as "non-binders." In this BRD, such substances are classified as negative in the ER binding assay conducted.

Because of the potential for variation in IC_{50} values among ER binding assays or repeats of assays that use different preparations of ER protein, the generally accepted method for presenting and comparing assay results is to compute the relative binding affinity (RBA) of the test substance against a reference estrogen. The RBA is calculated as $IC_{50(\text{reference estrogen})}/IC_{50(\text{test substance})} \times 100$. 17β-Estradiol is generally used as the reference estrogen for calculating the RBA value, but diethylstilbestrol (DES) has also been used. Because RBA values cover approximately eight orders of magnitude and because there is no current guidance as to which levels of activity are biologically meaningful in terms of an adverse health outcome, there is no general agreement regarding the distinction between the values needed to distinguish endocrine disruptors from non-disruptors.

1.2.3 Mechanistic Basis of *In Vitro* ER Binding Assays

The ER is a transcriptional regulatory protein belonging to the nuclear hormone receptor superfamily. The receptor is localized in the soluble nuclear fraction of estrogen target cells and plays a major role in controlling the transcriptional activation and/or repression of estrogen-responsive genes. The ER contains two discrete domains that are necessary for its role as a transcription factor – a ligand-binding domain in the *C*-terminal region and a DNA-binding domain in the *N*-terminal region of the protein. The ligand-binding domain, which is contained

within a wedge-shaped cavity on the receptor, is relatively hydrophobic. This allows the ligand-binding domain to accommodate its endogenous, nonpolar ligand, 17β-estradiol. The DNA-binding domain contains a zinc finger motif found in many DNA-binding proteins (Kumar et al., 1987; Brzozowski et al., 1997).

Recently, a second subtype of the ER, termed ERβ, has been identified (Kuiper et al., 1997). The classical ER is now termed ERα. Many similarities exist between the two subtypes. The DNA-binding domains have about 97% amino acid homology, while the ligand binding domains have about 60% homology (Kuiper and Gustafsson, 1997). Because of these similarities, ERα and ERβ share similar binding kinetics for many but not all of the estrogenic compounds tested with both subtypes. The two subtypes have unique tissue distributions, different physiological roles, and differ in their modes of regulating gene transcription (Kuiper et al., 1998; Gaido et al., 1999).

As the primary receptor for endogenous estrogens that initiate the transcription of messenger RNA and ultimately protein synthesis in estrogen-target cells, the ER plays a pivotal role in the development and maintenance of the female reproductive system. The interaction of estrogens with the ER in a cell initiates a cascade of events, including the dissociation of corepressor proteins from the ER and the induction of significant, conformational changes in the receptor that allow the binding of coactivator proteins. This activated receptor complex binds to specific DNA regulatory sequences of estrogen-responsive genes (estrogen response elements; ERE) that are located upstream from or within the intron regions of the responsive genes. This binding initiates or inhibits the transcription of estrogen-controlled genes, which leads to the initiation or inhibition of cellular processes, respectively, including those necessary for cell proliferation, normal fetal development, or adult homeostasis (Kumar et al., 1987; Brzozowski et al., 1997; Love et al., 2000).

The current hypothesis for ER-mediated endocrine disruption is that certain xenobiotic substances, by virtue of their structure or conformation, bind to the ER and either mimic or block the action of 17β-estradiol. The ER system is a prime candidate for interference by xenobiotic substances because the ligand-binding domain of the ER is much larger than the space occupied

by 17β-estradiol, making the binding site somewhat nonspecific. This nonspecificity has been confirmed by studies demonstrating that a variety of different xenobiotic substances belonging to many structural classes bind to the ER (Blair et al., 2000; Pike et al., 2000; Fang et al., 2001). In addition, some substances, known as selective ER modulators (SERMs), cause the receptor to take on a conformation that is neither fully active nor inactive. SERMs have the ability to act as agonists in some estrogen-responsive tissues and as antagonists in others (McDonnell, 1999).

Potential agonist or antagonist estrogenic activity may be inferred for a substance by its ability to compete with 17β-estradiol for binding to the ER. *In vitro* ER binding assays have been proposed as predictors of estrogen disruption in intact organisms (U.S. EPA, 1997; 1998a,b; 1999). The validity of the binding assay results for this purpose requires a determination that the substance also elicits similar responses in an *in vivo* assay. Such concordance for several substances has been reported by Shelby et al. (1996).

Factors that affect ligand binding to the ER are:

- Affinity for the ER. This affinity depends on the rates of the association and disassociation of the ligand with the receptor. The natural ligand, 17β-estradiol, has a low equilibrium constant because of its rapid association rate and relatively slow disassociation rate. The half-life of the disassociation of 17β-estradiol with the ER in intact rat uterine cells has been reported to be 90 minutes (Kassis et al., 1986).
- Systemic half-life of the ligand. This half-life will depend on its rate of metabolism to an intermediate that binds or does not bind to the receptor, and to the clearance of the ligand and its metabolites from the organism.
- Concentration of the ligand. Weakly binding ligands can produce a biological effect if they are administered at high enough concentrations, and strongly binding ligands would be ineffective if they do not reach estrogen-sensitive tissues.

1.2.4 Relationship of Mechanisms of Action in *In Vitro* ER Binding Assays Compared to the Species of Interest

Although the ER system is highly conserved among vertebrate species, and substances binding to ER derived from one species are expected to bind to the ER from another vertebrate species, the

relative binding affinities of these receptors for the same ligand may be different. Currently, little is known about the comparative binding of ligands to the ER of different species (Ankley et al., 1998). However, the ER from the rainbow trout has been reported to differ both structurally and functionally from its counterpart in mammals (Petit et al., 1995). In this regard, Zacharewski and coworkers (Matthews et al., 2000; Matthews and Zacharewski, 2000) recently showed that polychlorinated biphenyls (PCBs) have more affinity for the binding domain of rainbow trout ER (rtER) than to the binding domains of ERs from human, rat, mouse, or amphibian. Despite these differences and due to a lack of information on interspecies comparisons, the present working hypothesis is that the biological effects in one vertebrate species resulting from exposure to an endocrine disruptor is presumed to occur in other species. This approach is the basis for the use of ER binding as a general screen for estrogenic effects. The most widely used ER binding assays use human or rat ER-containing cells, or cytosolic ER derived from human or rat cells or tissues. Substances that bind the ER from these cells and tissues are presumed to be capable of producing estrogenic effects in multiple species. However, there is insufficient evidence to demonstrate that this extrapolation is appropriate. It is also not known whether differences in ER ligand affinity between species are meaningful with regard to *in vivo* adverse effects.

1.3 Intended Uses of the Proposed *In Vitro* ER Binding Assays

In vitro ER binding assays are proposed components of the EDSP Tier 1 screening battery. The Tier 1 battery is comprised of multiple *in vitro* and *in vivo* assays that assess both receptor- and nonreceptor-mediated mechanisms of action and endpoints. This battery is designed to detect substances that might affect estrogen, androgen, and thyroid hormone systems in multiple species, including humans.

1.3.1 Validation of *In Vitro* Assays

The FQPA requires the U.S. EPA base its endocrine disruptor screening program on validated test systems, and that the assays selected for inclusion in the program be standardized prior to their adoption. The ICCVAM Authorization Act (Sec. 4(c)) mandates that "[e]ach Federal Agency … shall ensure that any new or revised … test method … is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use]." (P.L. 106-545,

2000). The validation process will provide data and information that will allow the U.S. EPA to develop guidance on the development and use of functionally equivalent assays and endpoints prior to the implementation of the screening program.

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM, 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM, 1997). For the *in vitro* ER binding assays described in this BRD, relevance is restricted to how well an assay identifies substances that are capable of binding to the ER. The reliability of an assay is defined as its intra- and inter-laboratory reproducibility. Both relevance and reliability should be based on a diverse set of substances representative of the types and range of responses expected to be identified.

The first stage in assessing the validation status of an assay is the preparation of a BRD that presents and examines the available data and information about the assay, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM, 1997). This BRD summarizes the available data and information on the various types of *in vitro* ER binding assays that have been commonly used to characterize substances as potential endocrine disruptors. Where appropriate data are available, the qualitative and quantitative performance characteristics of the assays are evaluated, and the reliability of each assay is compared with the reliability of the other assays. These evaluations are used to determine whether a specific assay or assay type (e.g., whole cell, cell cytosol, tissue cytosol, recombinant ER) has been validated sufficiently to allow its recommendation for adoption by the U.S. EPA as an EDSP Tier 1 assay. If there are insufficient data to support the recommendation of an assay, this BRD will aid in identifying which specific assays should undergo further development or validation. The analyses can also be used to identify minimum procedural standards that should be considered for current and future *in vitro* ER binding assays.

1.3.2 Where Can *In Vitro* ER Binding Assays Substitute, Replace, or Complement Existing Methods?

There are no *in vitro* assays for ER binding or TA that are currently accepted by regulatory agencies. The *in vitro* ER binding assays are intended, along with other *in vitro* and *in vivo* tests, to be a component of the proposed EDSP Tier 1 battery for identifying endocrine disruptors.

1.3.3 Similarities and Differences with Currently Used Methods

The measurement of ER binding activity *in vitro* is not currently required for regulatory decision-making. However, there are a number of *in vitro* assays available for measuring receptor binding. These assays are based on the same general principles, but may use different sources of ER and different protocols.

The most frequently used ER binding assays use uterine cytosol from rats and mice as the source of the ER. Cytosol from other sources, such as the breast cancer cell line MCF-7, has also been used. Assays using purified receptor proteins have recently been introduced following the identification of the ERα and ERβ receptors from different tissues. Relative binding of a ligand with the receptor has been measured using either radiolabeled 17β-estradiol or by FP techniques.

1.3.4 Role of *In Vitro* ER Binding Assays in Hazard Assessment

The *in vitro* ER binding assays are proposed as a component of the proposed EDSP Tier 1 screening battery that also includes androgen receptor binding assays, *in vitro* ER and AR TA assays, and *in vivo* assays for endocrine effects in rodents, amphibians, and fish. The EDSTAC committee recognized that TA assays provide more information than binding assays because they measure also the consequences of binding. However, the limited databases at that time did not allow a determination of whether one or the other, or both assays, were preferred for screening (U.S. EPA, 1998a). Subsequently, the EDSP expressed a preference for TA assays over receptor binding assays because these assays can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation (U.S. EPA, 1999).

The assays in the Tier 1 battery have been combined in a manner such that limitations of one assay are complemented by strengths of another. The *in vitro* assays measure the interactions

between the test substance and binding and/or transcriptional activation only, and may therefore produce false positive results that may not occur *in vivo* due to limited absorption, distribution, metabolism, and excretion of the substance. The *in vitro* assays may also produce false negative results due to the absence of active metabolites that are formed *in vivo,* and to endocrine-related effects that are mediated by mechanisms not addressed by the *in vitro* assays.

A positive result in the ER binding assay or in other Tier 1 screening assays would not be sufficient to make the determination that a substance would produce a hormone-related adverse health effect in humans or other species. A weight-of-evidence approach will be used to evaluate the battery of Tier 1 results and to make decisions about whether or not a test substance would be subject to Tier 2 testing (U.S. EPA, 1998b). The Tier 2 assays are all performed *in vivo* and were selected to determine if a substance identified in Tier 1 as a potential endocrine disruptor exhibits endocrine-mediated adverse effects in animals and to identify, characterize, and quantify these effects.

1.3.5 Intended Range of Substances Amenable to *In Vitro* ER Binding Assays and/or Limits of *In Vitro* ER Binding Assays

The range of substances amenable to testing in *in vitro* ER binding assays has yet to be determined and will depend on the outcome of an independent peer review of the assays considered in this BRD. The *in vitro* ER binding assays are intended to be used to test food components and contaminants, as described in the FQPA (P.L. 104-170), and water contaminants, as described in the 1996 Amendments to the SDWA (P.L. 104-182). In addition, the U.S. EPA has authority to test commercial substances regulated by the Toxic Substances Control Act (TSCA, 1976) in the following circumstances: 1) the SDWA provides for testing of TSCA substances present in drinking water; 2) the FQPA amendments and the Federal Food Drug and Cosmetic Act (FFDCA; 21 CFR Ch.9., 1996) provide for testing of "inerts" in pesticide formulations; and 3) the FQPA and FFDCA provide for testing of substances that "act cumulative to a pesticide."

1.4 Search Strategy and Selection of Citations for the *In Vitro* ER Binding BRD

The *in vitro* ER binding data summarized in this BRD are based on information found in the peer-reviewed scientific literature. An online literature search of entries in MEDLINE, CANCERLIT, TOXLINE, AGRICOLA, NIOSHTIC, EMBASE, CABA, BIOSIS, and LifeSci was conducted to retrieve database records on publications reporting on *in vitro* testing of substances for their endocrine disrupting effects. The search was conducted in the database basic index, which includes words in the title and abstract, and indexing words. Specifically, records on estrogen/androgen receptor binding assays and estrogen/androgen TA assays were sought. The search strategy involved the combining of "*vitro*" with alternative terms for estrogens, androgens, receptors, binding, and testing. Each database record included authors, bibliographic citation, and indexing terms. Most records also included abstracts.

Of the 459 records obtained from the initial search conducted on December 12, 2000, 354 contained data from estrogen-related assays and 105 contained data from androgen-related assays. Abstracts of selected titles were reviewed, and the relevant articles were selected and retrieved from the literature for analysis. A database of the literature citations was established using bibliographic database software. Subsequent to the initial search, additional articles with relevant information were identified and retrieved; many of these were identified from the bibliographies of the previously selected articles. Scanning of the literature using *Current Contents* and the British Lending Library's *Table of Contents* continued through the writing of this BRD, and recently published articles were added to the database as they became available. Identification of ER-related publications for data extraction was completed on September 30, 2001.

The most relevant reports were those containing data on substances that have been tested in more than one laboratory using identical or related protocols. Every effort was made to include data from these publications because they provided information that could contribute to the assessment of the performance and reliability of the different assays. Publications containing data for substances that were synthesized specifically for the reported study and were not tested in other laboratories or in other *in vitro* ER binding assays did not contribute to the analysis of the data for performance and reliability. Primarily, these studies compared the binding affinities

of structural and positional isomers of known binding agents (such as 17β-estradiol) that were synthesized specifically for the study and are not available commercially. Data on the ER binding affinity of some of these substances are included in the BRD. Data was not extracted from reports of studies using a unique procedure or from studies that tested obscure or difficult-to-identify substances. Based on these criteria, data from 72 publications was abstracted and included in this BRD.

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